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Clonal Lymphoproliferation Following Chronic Active Epstein-Barr Virus Infection and Hypersensitivity to Mosquito Bites

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In order to elucidate the possibility of lymphoproliferation in cases of chronic active Epstein-Barr virus infection (CAEBV), to clarify the clonality and genotype of proliferating lymphocytes, and to search for the factors that induce lymphoproliferation, we studied 11 cases of CAEBV, using genetical and immunological techniques.

Epstein-Barr virus (EBV) DNA in peripheral mononuclear cells was detected in eight cases by Southern blotting. Among those eight cases, monoclonal proliferation of EBV DNA-positive cells was observed in three cases and oligoclonal proliferation in three cases. In the cases of monoclonal proliferation, one case manifested T-cell lymphoproliferation and the rest natural killer (NK) cell lymphoproliferation. The anti-EBV antibody titers in the study did not have any relativity to lymphoproliferation. On the other hand, three of the four cases of NK cell lymphoproliferation and one of the two cases of T-cell lymphoproliferation exhibited hypersensitivity to mosquito bites (HMB) in their clinical histories, while none of the three nonlymphoproliferation cases did.

These facts indicate that T-cell and NK cell lymphoproliferative diseases (LPDs) could be more closely associated with EBV infection than we had previously expected. Also, the anti-EBV antibody titers may not be the indicator of EBV-associated LPD, and HMB may be one of the factors that induce EBV-associated LPD. Am. J. Hematol. 54:276–281, 1997. © 1997 Wiley-Liss, Inc.

Key words: Epstein-Barr virus (EBV); chronic active Epstein-Barr virus infection (CAEBV); lymphoproliferative disease (LPD); hypersensitivity to mosquito bites (HMB)

INTRODUCTION

Recently, it has been recognized that Epstein-Barr virus (EBV) can infect and proliferate T cells and natural killer (NK) cells in addition to B cells among lymphocytes. On the other hand, the clinical entity of chronic active Epstein-Barr virus (CAEBV) infection has been established [1–6]. The diagnostic criteria of CAEBV proposed by Rickinson are as follows: (1) chronic or recurrent infectious mononucleosis-like symptoms lasting for a period of at least 1 year and often longer, (2) an unusual pattern of anti-EBV antibodies with raised anti-early antigen (EA) and/or absent anti-EBV-associated nuclear antigen (EBNA) titers, and (3) no evidence of any prior immunological abnormality or of any other recent infection by which to explain the condition [7].

In this condition, since the lymphocytes of patients may encounter continuous exposure to EBV, they could have a high probability of being infected and proliferated by the virus. Indeed, we have observed lymphocytoses or sequent lymphoproliferative diseases (LPDs) in cases of

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TABLE I. Profile of Patients and Serological Data at Diagnosis

Case	Sex	Age at onset	Age at study	VCA			EA-DR			EBNA	Clinical history
				IgG	IgA	IgM	IgG	IgA	IgM		
1	M	8	11	2,560	ND	10	1,280	ND	ND	40	HMB
2	M	1	9	10,240	640	<10	1,280	80	ND	640	NP
3	F	9	10	640	<10	<10	1,280	<10	<10	80	HMB
4	M	10	13	1,280	<10	<10	320	<10	ND	40	HMB
5	M	7	12	640	<10	40	320	40	ND	40	NP
6	M	2	5	640	ND	ND	80	<10	ND	160	NP
7	F	7	11	2,560	160	20	640	ND	ND	160	NP
8	M	9	12	320	10	10	80	<10	ND	10	HMB
9	F	6	9	640	<10	<10	10	<10	ND	40	NP
10	M	5	8	640	80	<10	320	ND	ND	160	HMB
11	M	9	12	320	40	20	160	40	ND	640	HMB

VCA, viral capsid antigen; EA-DR, early antigen diffuse or restricted pattern; EBNA, EB virus-associated nuclear antigen; HMB, hypersensitivity to mosquito bites; NP, nothing particular; ND, not done.

CAEBV [8–11]. But these lymphocytoses or LPDs have not been observed in most CAEBV cases [6].

From November 1985 to February 1992, we experienced 11 cases of CAEBV. To investigate the possibility of lymphocytosis or LPD in the cases of CAEBV, to clarify the clonality and genotype of the proliferating lymphocytes and to search for the factors that induce lymphocytosis or LPD, we studied these cases from the hematological, serological, and genetic points of view.

MATERIALS AND METHODS

Patients and Samples

The profile of the patients included in the study is shown in Table I. Rickinson's criteria were fulfilled in all cases. All were from the Western area of Japan, but they were not related to one another. As regards their clinical histories, while no patients had been diagnosed with immunodeficiencies or LPD, interestingly, six patients (cases 1, 3, 4, 8, 10, and 11) had hypersensitivity to mosquito bites (HMB), characterized by intense local skin symptoms and high fever following the bites.

The blood samples were obtained at random, that is, without regard to the count of peripheral blood lymphocytes. Four cases (4, 8, 9, and 11) revealed granular lymphocytosis, and one case (2) revealed absolute lymphocytosis at the time of this study. Lymphocytosis was not manifested in the rest of the cases (Table II).

The phenotype of peripheral blood lymphocytes are shown in Table II. $CD4^+$ cells increased ($\geq$60%) in cases 2 and 10. $CD16^+$ cells increased ($\geq$30%) in cases 4 and 8. $CD38^+$ cells increased ($\geq$20%) in cases 4, 5, 8, 9, and 11; HLA-DR^+ cells increased ($\geq$50%) in cases 1, 2, 4, 8, 9, 10, and 11.

Immunological studies showed that all cases were proved not to be affected by cytomegalovirus (CMV), human T-lymphotropic virus-I (HTLV-I), or human immunodeficiency virus (HIV).

Southern Blotting

To investigate the presence of EBV DNA and human herpesvirus-6 (HHV-6) DNA in the proliferating lymphocytes and to clarify the clonality and genotype of the lymphocytes, we carried out Southern blotting. DNA was extracted from the peripheral blood mononuclear cells stored at −80°C; 5 μg of DNA was digested with 50 units of restriction endonuclease, *Bam*HI or *Eco*RI, at 37°C for 3 hr. The digested DNA was subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon filter using the Southern blotting technique [12]. Filter-bound DNA fragments were hybridized with ^{32}P-labeled probes and visualized on an autoradiogram.

As for the probes, we used *Bam*HI-W fragments to investigate the presence of EBV DNA [13]. With the samples in which EBV DNA had been detected, we used the terminal repeat sequences, known as termini [14], to clarify the clonality of EBV DNA-positive cells. To investigate the presence of HHV-6 DNA, we used HHV-6 *Eco*RI D and E fragments (provided by Professor K. Yamanishi, Research Institute for Microbial Disease, Osaka University). In addition, we used the immunoglobulin (Ig) heavy chain J_H gene probe [15] and the T-cell receptor (TcR) $C_\beta 1$ gene probe [16] to investigate the genotype and clonality of proliferating cells.

Anti-EBV antibody titers

To investigate the relation between lymphoproliferation and anti-EBV antibody titers, we simultaneously examined the antiviral capsid antigen (VCA) IgG, IgA and IgM, anti-EA IgG, IgA and IgM, and anti-EBNA in the serum, which had been stored at −20°C using the fluorescent antibody method.

RESULTS

While HHV-6 DNA was not detected in all 11 cases (data not shown), EBV DNA was detected in eight cases

TABLE II. Hematological Data for Patients in This Study

	Case										
	1	2	3	4	5	6	7	8	9	10	11
WBC	1,500	19,200	5,200	4,000	6,000	5,700	3,800	10,600	3,900	9,100	3,500
St	13	0	2	0	7	4	10	2	27	5	4
Seg	49	6	52	23	47	20	56	14	33	25	25
Ba	0	0	1	1	4	3	2	3	0	1	0
Eo	0	0	2	1	0	0	0	0	0	0	1
Ly	22	86	39	23	35	55	23	25	15	67	8
Mo	16	0	3	2	7	18	9	6	5	1	8
AL	0	8	1	0	0	0	0	0	2	1	2
GL	0	0	0	50	0	0	0	50	18	0	52
CD 2	ND	ND	ND	78.7	ND	ND	48.0	89.0	67.5	ND	78.0
CD 3	86.1	98.3	51.7	27.4	74.9	72.4	47.3	29.8	18.6	ND	15.0
CD 4	43.5	90.1	38.0	22.5	31.6	34.4	31.0	19.4	10.5	68.5	6.4
CD 8	32.8	11.0	21.1	12.8	35.2	28.2	17.6	13.2	34.8	14.9	8.0
CD16	ND	ND	ND	35.2	6.4	5.6	3.7	50.4	15.6	ND	ND
CD20	8.2	1.0	7.1	12.3	18.9	ND	ND	ND	9.8	ND	ND
CD38	ND	ND	ND	60.3	30.6	ND	15.2	50.5	45.0	ND	34.0
CD57	ND	ND	ND	1.0	13.5	12.8	11.2	4.2	3.4	2.5	5.0
DR	61.3	92.1	8.3	86.4	15.7	ND	35.6	74.1	65.2	72.3	50.0

WBC, white blood cells; St, staff cells; Seg, segmentocytes; Ba, basophilic leukocytes; Eo, eosinophilic leukocytes; Ly, lymphocytes; Mo, monocytes; AL, atypical lymphocytes; GL, granular lymphocytes; CD, cluster of differentiation; DR, HLA-DR; ND, not done.

(1, 2, 3, 4, 8, 9, 10, and 11) (Fig. 1). The clonality of EBV DNA-positive cells was assessed for the eight cases (Fig. 2, Table III). This examination showed that the EBV DNA-positive cells in cases 2, 8, and 9 were monoclonal, those in cases 4, 10, and 11 were oligoclonal, and those in case 1 and 3 might be polyclonal.

The results of DNA analysis on the Ig gene and the TcR gene are also shown in Table III. While rearrangement of the J_H gene was not detected in all examined cases, that of the $C_\beta 1$ gene was recognized in case 2.

These data indicate that the monoclonally proliferating EBV-positive cells in case 2 may have developed from T cells, and those in cases 8 and 9 may have developed from NK cells. Moreover, the oligoclonally proliferating EBV-positive cells may have developed from T cells or NK cells, perhaps from NK cells in cases 4 and 11, and from T cells in case 10, per the outcome of morphological and immunological studies.

The serological data are shown in Table IV. Interestingly, while 10 cases presented positive titers of anti-EA IgG ranging from ×10 to ×320, only case 2 showed a negative titer.

Three cases (5, 6, and 7) that did not manifest clonal lymphoproliferation have not revealed overt lymphocytosis or LPD heretofore.

Seven of these 11 patients (cases 1, 2, 4, 7, 9, 10, and 11) died within 1 month to 3 years of this study. None of these cases had been diagnosed with a hematological malignancy in their clinical course, except for peripheral lymphocytosis. In each case, the causes of death was not LPD or hematological malignancy, but organ failure or sepsis. Four of these patients died of hepatic failure, and the remainder died of cardiac failure, pulmonary failure, or sepsis.

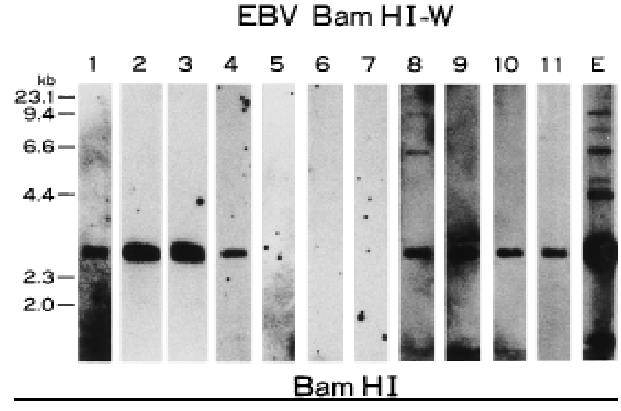

Fig. 1. DNA analysis of the peripheral blood mononuclear cells with *Bam*HI-W fragments as the probe. The band was detected at 3 kb in eight cases. These results indicated that a large amount of EBV DNA had penetrated the mononuclear cells in these eight cases. E, EBV DNA-positive cells (Raji cells).

DISCUSSION

Our data indicate that EBV can infect T cells and peripheral lymphoproliferation of $CD3^+$ cells may occur under CAEBV. Similarly, our data indicate that EBV can infect NK cells and may induce NK cell lymphoproliferation in patients with CAEBV.

Moreover, our data indicate that lymphoproliferation of EBV DNA-positive cells can be observed in cases of

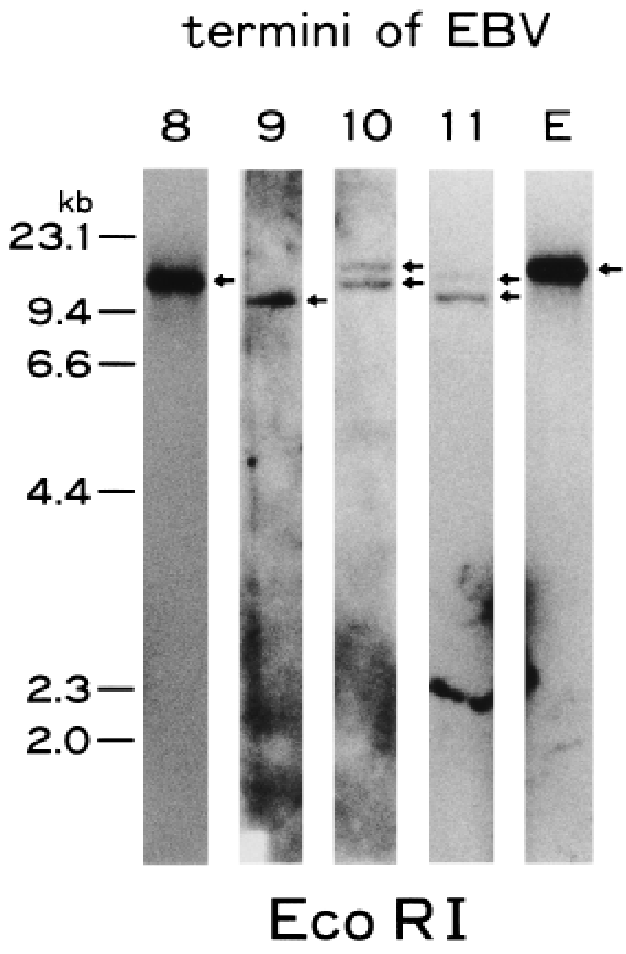

Fig. 2. DNA analysis of the peripheral blood mononuclear cells with terminal repeat sequence as the probe. One band was detected in cases 8 and 9, and two bands were detected in cases 10 and 11. These results indicated that EBV-positive cells in cases 8 and 9 had monoclonally proliferated and that those in cases 10 and 11 had oligoclonally proliferated. E, EBV DNA-positive cells (Raji cells), proliferating monoclonally.

CAEBV in whom lymphocytosis is not manifested such as cases 1, 3, and 10. These facts suggest that some cases of CAEBV without lymphocytosis may conceal lymphoproliferation.

These patients with CAEBV have been continuously affected by EBV. If chronic active or continuous EBV infection can induce peripheral lymphoproliferation of B cells, T cells or NK cells, all cases in our study could have manifested lymphoproliferation. However, three cases (5, 6, and 7) manifested neither lymphoproliferation in our study nor lymphocytosis in their clinical course. In addition, only 13 cases had manifested LPDs in their clinical course among 39 cases of CAEBV in Japan [6]. These facts suggest that chronic active or continuous EBV infection may not induce lymphoproliferation by itself.

If the activity of EBV is responsible for the lymphoproliferation, the anti-EBV antibody titers, especially anti-VCA IgG or anti-EA IgG, may have some relativity to lymphoproliferation. However, as shown in Table IV, there was no relationship between them.

TABLE III. Results of Southern Blotting

Case	*Bam*HI-W	Termini	TcR (C_β)	Ig (J_H)
1	+	—	G	G
2	+	S	R+R/G	G
3	+	—	G	G
4	+	D	G	G
5	−	—	ND	ND
6	−	—	ND	ND
7	−	—	ND	ND
8	+	S	G	G
9	+	S	G	G
10	+	D	G	G
11	+	D	G	G

S, single band; D, double band; G, germline; R, rearrangement; ND, not done.

Many cases of T-cell lymphoma or NK cell lymphoproliferation, in which the presence of EBV DNA had been proved in the proliferating cells, were reported in Japan [17,18] and Taiwan [19,20]. While some of them had followed CAEBV, the anti-EBV antibody titers were not specific but were positive in most cases. These findings also suggest that lymphoproliferation may not be induced by active EBV infection, but by some other factors associated with EBV infection.

One of the candidates for the factor is double infection with other herpesviruses or retroviruses. However, as mentioned under Materials and Methods, and Results, CMV, HTLV-I, HIV, and HHV-6 may have no effect on lymphoproliferation.

The recent literature on newly reported cases of EBV-associated LPD reflects publications from all over the world. While most cases of Hodgkin's disease have been reported from Europe or the United States [21,22], most cases of T-cell lymphoproliferative disease (TLPD) and granular lymphocytes proliferative disorder (GLPD) have been reported from Asia, especially East Asia, Taiwan [19,20], and Japan [11,17,18]. These facts suggest that TLPD and GLPD reported from Taiwan and Japan may be induced by EBV and by some environmental or racial factors. We surmise that the environmental factors may include climate, animals, plants, water, and so on, and that the racial factors may include ethnicity and type of histocompatible leukocyte antigen.

Although we must investigate these candidates individually, we have noticed in our study that one of the possible factors is the clinical history of HMB. While three patients who did not manifest lymphoproliferation did not exhibit this history of HMB, four of six patients who did manifest monoclonal or oligoclonal lymphoproliferation had HMB.

TABLE IV. Anti-EBV Antibody Titers of the Patients in This Study

	VCA			EA-DR			
Case	IgG	IgA	IgM	IgG	IgA	IgM	EBNA
1	640	<10	<10	160	<10	<10	10
2	640	80	<10	<10	10	<10	20
3	160	<10	<10	80	<10	<10	40
4	640	<10	<10	40	<10	<10	80
5	160	<10	<10	80	<10	<10	40
6	1,280	<10	<10	320	<10	<10	320
7	2,560	<10	<10	320	40	<10	40
8	640	10	20	20	10	<10	10
9	80	<10	<10	10	<10	<10	40
10	320	40	<10	160	10	<10	160
11	640	160	<10	160	40	<10	80

VCA, viral capsid antigen; EA-DR, early antigen diffuse or restricted pattern; EBNA, EB virus-associated nuclear antigen.

While the first case of HMB was reported from Florida in 1938 [23], subsequent cases have been reported from Japan [24] and Taiwan [25]. Moreover, patients with HMB have been assumed to be at risk of an LPD, such as hemophagocytic syndrome or GLPD [26–28]. In our other study of CAEBV in Japan, six of 13 (46.2%) patients with LPD had a history of HMB, as opposed to only one of seven (14.3%) patients with cardiovascular disease, which is one of the major complications of CAEBV [6].

These facts suggest that HMB may be one of the factors that induce EBV-associated LPD. To clarify the mechanism by which EBV-associated LPD may be produced, we need to conduct a careful study of patients with CAEBV and HMB.

In conclusion, our data indicate that (1) patients with CAEBV in Japan are at higher risk of TLPD and GLPD than we had previously expected from the morphological point of view, (2) the anti-EBV antibody may not be the indicator of EBV-associated LPD, (3) one of the factors that induce EBV-associated LPD may be HMB, and (4) to clarify the mechanism that makes EBV-associated TLPD and GLPD, careful study of patients with CAEBV and HMB is needed.

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